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Gender hormones and the progression of experimental polycystic kidney disease

KENNETH D. STRINGER, RADKO KOMERS, SHUKRI A. OSMAN, TERRY T. OYAMA, JESSIE N. LINDSLEY, and SHARON ANDERSON

Division of Nephrology and Hypertension, Oregon Health and Science University, Portland, Oregon; Diabetes Center, Institute for Clinical and Experimental Medicine, Prague, Czech Republic; and Portland VA Medical Center, Portland, Oregon

Gender hormones and the progression of experimental polycystic kidney disease.

Background. Male gender is a risk factor for progression of autosomal-dominant polycystic kidney disease (ADPKD), clinically and in the Han:SPRD rat model. Orchiectomy limits progression, but mechanisms of the detrimental effect of androgen, and/or beneficial effects of estrogen, are not known. This protocol tested the hypothesis that male gender (intact androgen status) promotes progression, while female gender (intact estrogen status) is protective; and that these disease-modifying effects are due to changes in expression of known fibrotic mediators.

Methods. Studies were performed in male and female noncystic control (+/+) and cystic (+/−) rats subjected to orchiectomy, ovariectomy, or sham operation. At 12 weeks of age, renal function was measured. Blood and kidneys were taken for measurement of plasma and renal renin, endothelin (ET-1), endothelial nitric oxide synthase (eNOS), and vascular endothelial growth factor (VEGF), using biochemical, protein expression, and immunohistochemical methods.

Results. Cystic male rats exhibited significantly reduced glomerular filtration (GFR) and effective renal plasma flow (ERPF) rates, with suppression of plasma and renal renin, upregulation of renal ET-1 and eNOS, and down-regulation of renal VEGF expression. Orchiectomy attenuated the fall in GFR and ERPF, while numerically limiting changes in eNOS and VEGF. Female rats exhibited less cystic growth, with normal renin status, lesser elevation of renal ET-1, and proportionately lesser changes in VEGF and eNOS. Ovariectomy led to higher blood pressure and reduced GFR and ERPF, with a trend toward upregulation of ET-1, and significant down-regulation of VEGF and eNOS.

Conclusion. Female gender is protective, but ovariectomy attenuates the protective effect of female gender, in association with changes in renal expression of ET-1, VEGF, and eNOS. The accelerated disease in male rats can be attenuated by orchiectomy and consequent changes in expression of disease mediators.

Key words: gender, estrogen, polycystic kidney disease, VEGF, nitric oxide, endothelin.

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Gender is a determinant of progression of many forms of renal disease, with males exhibiting faster rates of progression to end-stage renal disease (ESRD). In autosomal-dominant polycystic kidney disease (ADPKD), recognized progression risk factors include diagnosis younger than age 30, gross hematuria at a young age, presence of the ADPKD1 gene, increased renal size, hypertension, and male gender [1, 2]. Affected men experience faster loss of renal function, and earlier onset of ESRD, than do women [1–7]. Experimentally, the Han:SPRD rat model shows similar gender dimorphism, in that females develop renal lesions only late in life [8–12]. Orchiectomy limits renal disease (assessed by renal size and cyst volume density), while testosterone replacement obviates the protective effect of gonadal ablation [10]. In females, testosterone increases kidney and cyst growth, with or without ovariectomy [10]. Together with the recent advances in understanding of the biology of sex hormones, these observations provide the rationale for study of mechanisms by which these hormones may affect the rate of progression.

These studies were designed to test several hypotheses related to sex hormones and the progression of cystic disease and renal insufficiency, in the Han:SPRD rat model of ADPKD. The foregoing observations suggested that the presence of male sex hormones (e.g., androgens), and/or deficiency of female sex hormones (e.g., estrogen), are associated with faster disease progression. A number of recent reports, primarily from in vitro studies, have indicated that 17β-estradiol and some of its endogenous metabolites exert potentially protective effects on the production, expression, and activity of various fibrotic mediators [13]. Though less well studied, androgens may exert specific deleterious effects. In this study, we sought to characterize the effects of gender and of gonadal ablation on the course of experimental PKD, and on the renal expression of potentially relevant disease mediators. This protocol tested the overall hypothesis that male gender (intact androgen status) promotes the progression of disease, while female gender (intact estrogen status) is

protective; and that this disease modification relates to altered expression or activity of known mediators of growth and fibrosis.

METHODS

These studies were conducted in Han:SPRD rats, from a colony propagated from breeding pairs kindly provided by Dr. Benjamin D. Cowley, Jr. (then at the University of Kansas Medical Center, Kansas City, KS, USA). Heterozygous cystic rats (Cy/+) and unaffected littermate control rats (+/+) were studied. All rats were fed standard rat chow (Rodent Laboratory Chow 5001) (Purina Mills, Richmond, IN, USA) ad libitum and had free access to water. These studies were approved by the Portland Veterans Affairs Institutional Animal Care and Use Subcommittee.

Male and female control [noncystic (+/+)] and heterozygous (Cy/+) rats were raised to the age of 6 weeks, at which time they underwent baseline measurements of body weight, awake systolic blood pressure, and 24-hour urine collections for measurement of proteinuria ($U_{prot}V$) and urinary nitrite + nitrate excretion ($U_{NOx}V$). Cystic males and females were then randomized to undergo orchiectomy or ovariectomy, or sham operation. They were then followed to the age of 12 weeks, at which time systemic measurements were repeated, and then some rats underwent renal function studies. In the remainder, after anesthesia with Brevital (50 mg/kg intraperitoneally), kidneys were removed for morphologic examination, protein expression studies, and immunohistochemical studies as detailed below. Blood was taken for measurement of plasma renin concentration (PRC), and kidneys were taken for measurements of renal content of renin (RRC) and endothelin-1 (ET-1). Other protein and immunohistochemical measurements are described below.

Renal function studies

Rats were anesthetized with Inactin (100 mg/kg intraperitoneally) and placed on a thermoregulated table. The left femoral artery was cannulated, and a baseline sample of blood was collected for determination of hematocrit and inulin and paraaminohippuriate (PAH) blanks. This arterial catheter was used for subsequent blood sampling and for estimation of mean arterial pressure (MAP) via an electronic transducer connected to a direct writing recorder. After tracheostomy, bilateral internal jugular catheters were inserted for infusions of rat serum and 10% inulin (Questcor, Carlsbad, CA, USA) and 1.0% PAH (Merck, West Point, PA, USA) in saline (1.2 mL/hour). To adjust for reduced renal clearances, cystic rats were given 6% inulin with 0.6% PAH in saline. The left ureter was catheterized for urine collections. To maintain euvolemia, rat serum was infused at 0.1 mL/min for a total equal to 1% of the body weight, followed by a reduction in infusion to 0.42 mL/hour, to maintain a constant hematocrit. After equilibration, triplicate 20-minute urine collections with midpoint blood collections were made, for measurement of hematocrit, inulin and PAH. Glomerular filtration rate (GFR) (by inulin clearance), effective renal plasma flow (ERPF) (by PAH clearance), filtration fraction (FF), and renal vascular resistance (RVR) were determined using standard formulas.

Biochemical studies

For calculation of GFR, inulin concentrations in plasma and urine were determined by the macroanthrone method. ERPF was determined by PAH clearance, with PAH concentrations in plasma and urine determined by colorimetric methodology. Plasma and renal renin concentrations were measured by radioimmunoassay using commercially available reagents (NEN Life Sciences, Boston, MA, USA). Tissue renin concentrations were expressed per mg protein, measured by BCA assay (Pierce Chemical Co., Rockford, IL, USA). Urine protein was measured by precipitation with 3% sulfosalicylic acid (Sigma Chemical Co., St. Louis, MO, USA). Renal ET-1 was determined via an enzyme-linked immunosorbent assay (ELISA) method adapted from Moreau et al [14] (Cayman Chemical, Ann Arbor, MI, USA). Urine NO_x was measured with a colorimetric assay (Cayman Chemical Co.).

Immunoblotting

Kidneys were homogenized in one of two buffers. For analysis of vascular endothelial growth factor (VEGF), total cell homogenates were generated in solubilization buffer [50 mmol/L Tris, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.0% Triton-X 100, and protease inhibitors (leupeptin, 20 μg/mL and benzamidine, 20 μg/mL)]. These homogenates were centrifuged at 12,000 × *g* for 30 minutes at 4°C and the supernatants were collected. For analysis of endothelial nitric oxide synthase (eNOS), cytosolic and crude-membrane preparations were generated in lysis buffer [25 mmol/L Tris, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), and protease inhibitors]. These preparations were centrifuged at 100,000 × *g* for 60 minutes at 4°C to obtain soluble (supernatant) and crude-membrane (pellet) fractions. Total protein content in fractions was determined by BCA analysis (Pierce Chemical Co.).

Immunoblotting was performed on each preparation as previously described [15]. In brief, denatured proteins were separated through an SDS-polyacrylamide gel and transferred to polyvinylidine difluoride (PVDF)

membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were washed and blocked overnight with Tris-buffered saline, 0.05% Tween-20 (TBST), containing 5% nonfat dry milk. Following blocking, membranes were again washed, and incubated overnight with a rabbit polyclonal antihuman VEGF antisera (A-20, SC-152) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (1:400) or with a mouse monoclonal anti-eNOS/NOS III antibody (#610296) (BD Transduction Laboratories, San Diego, CA, USA) (1:1000) diluted in TBST. Immunodetection was accomplished by incubating membranes with a goat antirabbit-IgG secondary antibodies conjugated with horseradish peroxidase (HRP) for 45 minutes (Pierce Chemical Co.) (1:100,000) in TBST containing 5% nonfat dry milk. Visualization was performed with enhanced chemiluminiscence (ECL) Western blotting kit (Supersignal West Dura) (Pierce Chemical Co.) according to the manufacturer's instructions. Resultant films (Eastman Kodak Co., Scientific Imaging Systems, New Haven, CT, USA) were scanned using a flatbed scanner and images analyzed with National Institutes of Health (NIH) Image software. The membranes were then stripped, reblocked, and reincubated for 1 hour at room temperature with goat antiactin antibody (Santa Cruz Biotechnology) (1:200), followed by 45-minute incubation with antigoat-IgG secondary antibody conjugated with HRP (1:4000) (Santa Cruz Biotechnology), and reaction with ECL as described above.

Immunohistochemistry

The same antisera as described above were used for immunohistochemical detection of VEGF and eNOS. Sections were deparaffinized in xylene, rehydrated through graded ethanols to water, and pretreated by steaming in 10% Citra buffer (BioGenex Laboratories, San Ramon, CA, USA). After being treated with protein-blocking solution, the slides were incubated overnight at 4°C with primary antibody (diluted 1:400 for VEGF and 1:1000 for eNOS) or with the same concentration of nonimmune IgG as a control. Endogenous peroxidase activity was blocked with 3% H_2O_2 solution in methanol. The primary antibody was localized using the Vectastain ABC-Elite peroxidase detection system (Vector Laboratories, Burlingame, CA, USA). This was followed by reaction with diaminobenzidine (DAB) as chromogen and counterstaining with hematoxylin (Sigma Chemical Co.). Sections of each polycystic kidney were processed in parallel with appropriate control tissue.

Morphologic studies

The left kidneys were immersed in 10% formalin, then dehydrated through a series of ethanols, embedded in paraffin, sectioned at 4 μm thickness, and placed onto glass slides. Cyst burden was quantified by a point counting method [16], on periodic acid-Schiff (PAS)–stained sections.

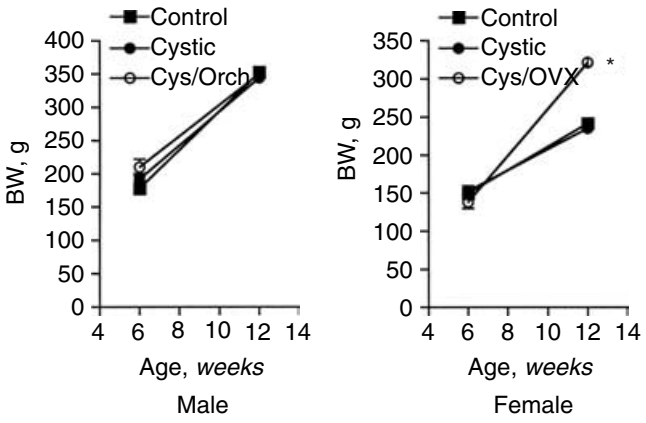

Fig. 1. Body weights in male and female control and cystic rats, with or without gonadectomy [orchiectomy (Orch) or ovariectomy (OVX)]. Males were heavier than females at both time points ($P < 0.01$). Body weights increased in all groups ($P < 0.01$ vs. 6 weeks of age). The cystic phenotype did not affect body weight. Orchiectomy had no effect on body weight in male rats, but ovariectomy significantly increased body weight gain in female rats ($P < 0.01$ vs. other female groups). * $P < 0.01$ vs. other female groups.

Statistics

Values are reported as mean ± SEM. Statistical analysis was performed by paired t test, or by analysis of variance (ANOVA) followed by computation of modified t values according to the method of Bonferroni (for multiple groups), as appropriate. Values which were not normally distributed were analyzed by nonparametric methods. Statistical significance was defined as $P < 0.05$.

RESULTS

Changes in body weight are depicted in Figure 1. Males were heavier than females at all time points, but the cystic state did not affect somatic growth in either gender. In cystic males, orchiectomy had no effect on body weight gain while in cystic females, ovariectomy resulted in accelerated weight gain ($P < 0.01$ vs. other female groups). Values for systolic blood pressure were similar in all groups at 6 weeks of age, except for a slight increment in cystic males as compared with noncystic control rats (Fig. 2). By 12 weeks of age, systolic blood pressure tended to be higher in cystic than in noncystic groups, but the differences were not statistically significant. Gonadal ablation did not affect systolic blood pressure in males, but significantly increased systolic blood pressure in cystic females at 12 weeks ($P < 0.05$). Results of proteinuria studies are shown in Figure 3. Values were low in all groups at 6 weeks of age, and did not differ with cystic phenotype, though baseline levels were higher in males than in females in all groups ($P < 0.05$). By 12 weeks of age, proteinuria had

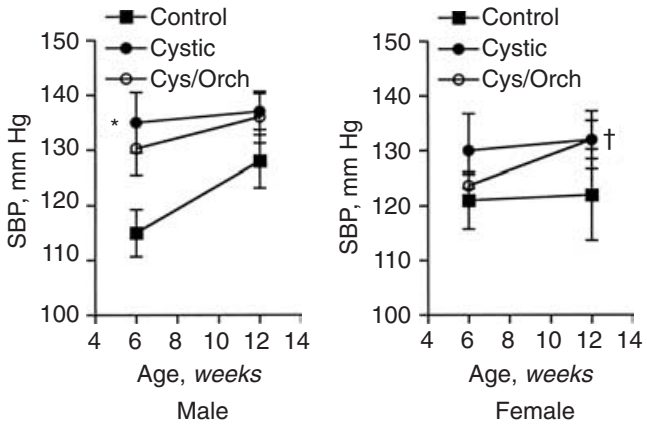

Fig. 2. Systolic blood pressures (SBP) in male and female control and cystic rats, with or without gonadectomy. Values for systolic blood pressure were similar in all groups at 6 weeks of age, except slightly higher in cystic than control males ($P < 0.05$). By 12 weeks of age, systolic blood pressure was numerically but not statistically higher in both male and female cystic rats, and systolic blood pressure rose significantly in cystic ovariectomy females ($P < 0.01$). *$P < 0.05$ vs. control males; †$P < 0.05$, Cys/ovariectomy, 12 weeks of age vs. baseline.

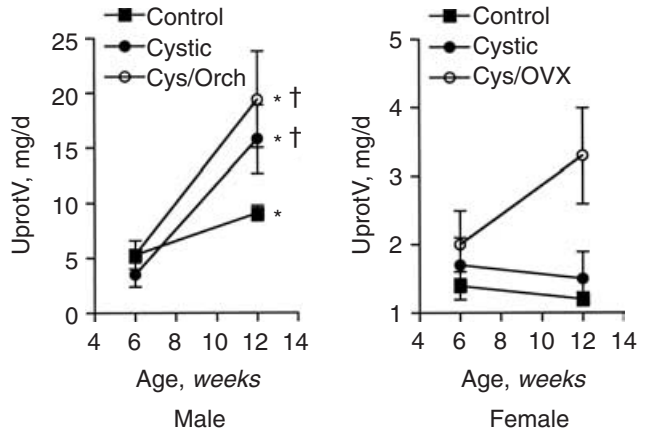

Fig. 3. Urinary protein excretion ($U_{prot}V$) in male and female control and cystic (Cys) rats, with or without gonadectomy [orchiectomy (Orch) and ovariectomy (OVX)]. Values for $U_{prot}V$ were low in all groups at 6 weeks of age, and significantly lower in females than in the corresponding males ($P < 0.05$). By 12 weeks of age, $U_{prot}V$ had increased significantly in male control ($P < 0.05$) and cystic ($P < 0.005$) rats, and orchiectomy did not attenuate the rise in proteinuria. Values in all female groups remained comparable, and lower than those in the male groups ($P < 0.05$). *$P < 0.01$ vs. baseline; †$P < 0.05$ vs. control males.

increased significantly in male control rats, but more so in male cystic rats, with or without orchiectomy. Proteinuria levels stayed low and near baseline in all female groups, with no significant change over time, though there was a trend toward increasing proteinuria in the ovariectomy females.

Results of somatic and renal growth and renal hemodynamic studies at 12 weeks of age are depicted in Table 1. As compared with noncystic control rats, the intact male cystic rats had comparable body weights at the time of experimentation, but markedly enlarged kidney weights and thus kidney body weight ratios ($P < 0.01$). As in our prior studies [16–18], the cystic males also exhibited modest elevations in MAP and decreased GFR and ERPF rates ($P < 0.05$), a trend toward elevation in FF, and increased values for renal vascular resistance (RVR) ($P < 0.05$ vs. controls). Orchiectomy did not affect body or kidney weight, but was associated with elevation of MAP as compared with the other male groups ($P < 0.05$). The orchiectomized male rats exhibited partially preservation of GFR and ERPF, both in absolute terms and when factored for body weight, as compared with intact cystic males ($P < 0.05$). Thus, orchiectomy was associated with preservation of renal function, despite a modest increment in blood pressure.

As compared with the corresponding males, the female control (noncystic) rats were smaller, with smaller kidney weights but comparable kidney body weight ratios. The female rats had lower GFR and ERPF rates than males ($P < 0.01$), although the differences were eliminated when factored for the lower body weights. Changes associated with cystic status were far less striking in the female than in the male rats. Cystic females had renal enlargement, but of significantly lesser magnitude than that in cystic males ($P < 0.01$). In contrast to the males, the cystic females had no impairment in GFR or ERPF, nor increment in RVR. Thus, cystic females rats exhibited protection against the structural and functional expression of the disease at this time point. However, functional and structural protection was abrogated with ovariectomy. The cystic kidneys in ovariectomized rats were 40% larger than those in intact female cystic rats ($P < 0.01$), though not statistically larger when factored for the difference in body weight. Functionally, when factored for body weight, ovariectomy led to a significant reduction in GFR and ERPF ($P < 0.05$). Thus, ovariectomy had the general effect of attenuating the protective effect of female gender on renal function.

Representative photomicrographs of renal cortex and medulla in male and female cystic rats are depicted in Figure 4. Cysts were prominent in both compartments in male rats, but less so in female rats. Quantitative assessment of cyst burden in intact and gonadectomized rats (Table 1) confirmed the lower cyst burden in the female groups, but found no significant effect of gonadal ablation on cyst burden.

Additional studies were performed to identify likely candidate mediators for the accelerated renal disease progression in the male rats (Fig. 5). As previously reported in this model [16], values for PRC were reduced in cystic males, as compared with noncystic controls; orchiectomy had no effect on PRC. In female rats, cystic disease did not result in any significant change in PRC. Values for RRC were slightly reduced in both intact and orchiectomized cystic males as compared with controls. In female rats, RRC did not differ among groups. Values

Table 1. Systemic and renal parameters

	Males			Females		
	Controls ($N = 12$)	Cystic ($N = 12$)	Cystic + orchiectomy ($N = 12$)	Controls ($N = 10$)	Cystic ($N = 12$)	Cystic + ovariectomy ($N = 10$)
Body weight *g*	374 ± 12	359 ± 6	361 ± 6	243 ± 2^{a}	242 ± 4^{a}	$321 \pm 6^{a,b,c}$
Left kidney weight *g*	1.48 ± 0.04	3.86 ± 0.14^{c}	3.73 ± 0.13^{c}	0.96 ± 0.02^{a}	$1.86 \pm 0.05^{a,c}$	$2.60 \pm 0.11^{a,b,c}$
Left kidney weight/100 g body weight	0.40 ± 0.01	1.08 ± 0.04^{c}	1.03 ± 0.04^{c}	0.39 ± 0.01	$0.77 \pm 0.02^{a,c}$	$0.81 \pm 0.04^{b,c}$
Mean arterial pressure *mm Hg*	127 ± 6	135 ± 2^{c}	$146 \pm 7^{b,c}$	119 ± 3^{a}	131 ± 5^{c}	$128 \pm 4^{b,c}$
Glomerular filtration rate *mL/min*	1.96 ± 0.08	0.77 ± 0.10^{c}	$1.10 \pm 0.09^{b,c}$	1.30 ± 0.08^{a}	1.31 ± 0.07^{a}	1.44 ± 0.10^{a}
Glomerular filtration rate/100 g body weight	0.53 ± 0.02	0.22 ± 0.03^{c}	$0.31 \pm 0.03^{b,c}$	0.53 ± 0.03	0.55 ± 0.03^{a}	$0.45 \pm 0.03^{a,b,c}$
Effective renal plasma flow *mL/min*	7.13 ± 0.31	2.63 ± 0.36^{c}	$3.59 \pm 0.29^{b,c}$	4.47 ± 0.27^{a}	4.21 ± 0.28^{a}	4.62 ± 0.36
Effective renal plasma flow/100 g body weight	1.93 ± 0.09	0.75 ± 0.11^{c}	$1.00 \pm 0.09^{b,c}$	1.84 ± 0.12	1.76 ± 0.12^{a}	$1.45 \pm 0.13^{a,b,c}$
Filtration fraction	0.28 ± 0.01	0.30 ± 0.01	0.31 ± 0.02^{c}	0.29 ± 0.01	0.32 ± 0.01^{c}	0.32 ± 0.01^{a}
Renal vascular resistance *mm Hg/mL/min*	10.6 ± 0.5	40.3 ± 6.9^{c}	28.7 ± 3.0^{c}	17.2 ± 1.3	20.6 ± 1.7^{a}	18.6 ± 2.0
Cyst burden%	–	20.3 ± 2.4	17.0 ± 0.9	–	6.6 ± 0.4^{a}	7.9 ± 0.5^{a}

Values are mean ± SEM.
[a] $P < 0.05$, female vs. respective male group; [b] $P < 0.05$ vs. respective intact group; [c] $P < 0.05$ vs. respective noncystic control.

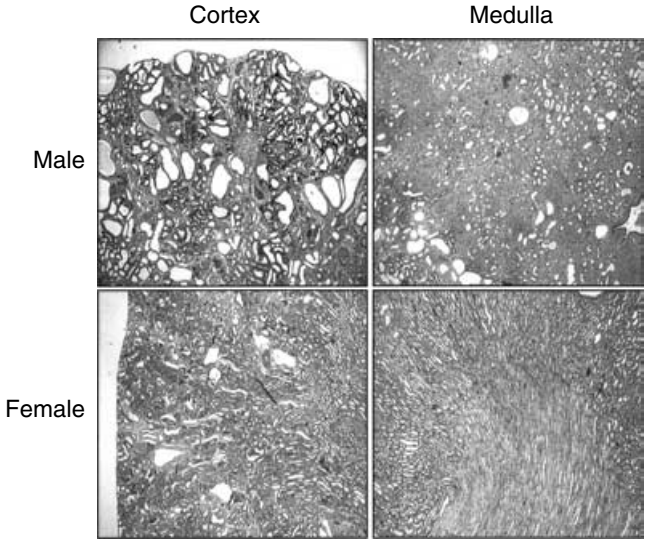

Fig. 4. Representative photomicrographs of renal cortex and medulla in male and female, cystic and noncystic rats. Cystic changes were apparent in both compartments in male rats, but were less prominent in female rats [40× periodic acid-Schiff (PAS) stain].

for renal ET-1 were markedly elevated in cystic male rats ($P < 0.01$), and unaffected by orchiectomy. In cystic female rats, renal ET-1 levels were significantly increased as compared with noncystic controls ($P < 0.05$), though not to levels seen in cystic males. Ovariectomy was associated with a trend toward further increased renal ET-1 values, to levels near those seen in cystic male groups.

Protein expression and immunohistochemical localization studies were used to examine renal expression of VEGF. Normalized VEGF protein expression was significantly reduced in cystic males, as compared with noncystic males (Fig. 6A) ($P < 0.05$). Noncystic female control rats demonstrated up-regulation of VEGF expression, as compared with noncystic males, whereas expression was also reduced in cystic females ($P < 0.05$). Orchiectomy slightly, though not significantly, increased VEGF expression in cystic males (Fig. 6B). In females, ovariectomy resulted in a further reduction in VEGF expression ($P < 0.05$). Immunohistochemical studies in noncystic control males revealed that VEGF expression was detected primarily in the thick ascending limb and distal tubular cells, in glomerular epithelial cells, and in vascular endothelia (Fig. 7). In the cystic male rats, VEGF was found mostly in cystic epithelial cells, and in atrophic tubules. Qualitatively, expression was similar in the males and females.

Renal expression of eNOS was also examined. As compared with noncystic males, renal eNOS was increased in male cystic kidneys ($P < 0.05$), and not significantly affected by orchiectomy (Fig. 8). Renal eNOS expression in noncystic females was higher than that in the respective male group. Expression of eNOS was up-regulated in the kidneys of cystic female rats, as compared with noncystic controls, but then reduced by ovariectomy. In immunohistochemical studies (not shown), eNOS displayed a diffuse granular pattern in tubular cells, in both cystic and noncystic rats. Immunoreactivity was also clearly detectable in arterial and arteriolar endothelial cells and to a lesser extent, in endothelia of glomerular capillaries, as previously reported [18]. Immunohistochemical analysis did not provide qualitative differences between the cystic and control groups, nor between the various groups. In addition, measurements of $U_{NOx}V$ did not show any consistent differences among groups (data not shown).

DISCUSSION

Gender as a risk factor

Male gender has recently been recognized as a risk factor for acceleration of clinical renal disease, especially in nondiabetic forms of chronic kidney disease. Men with ADKPD exhibit faster rates of progression and earlier

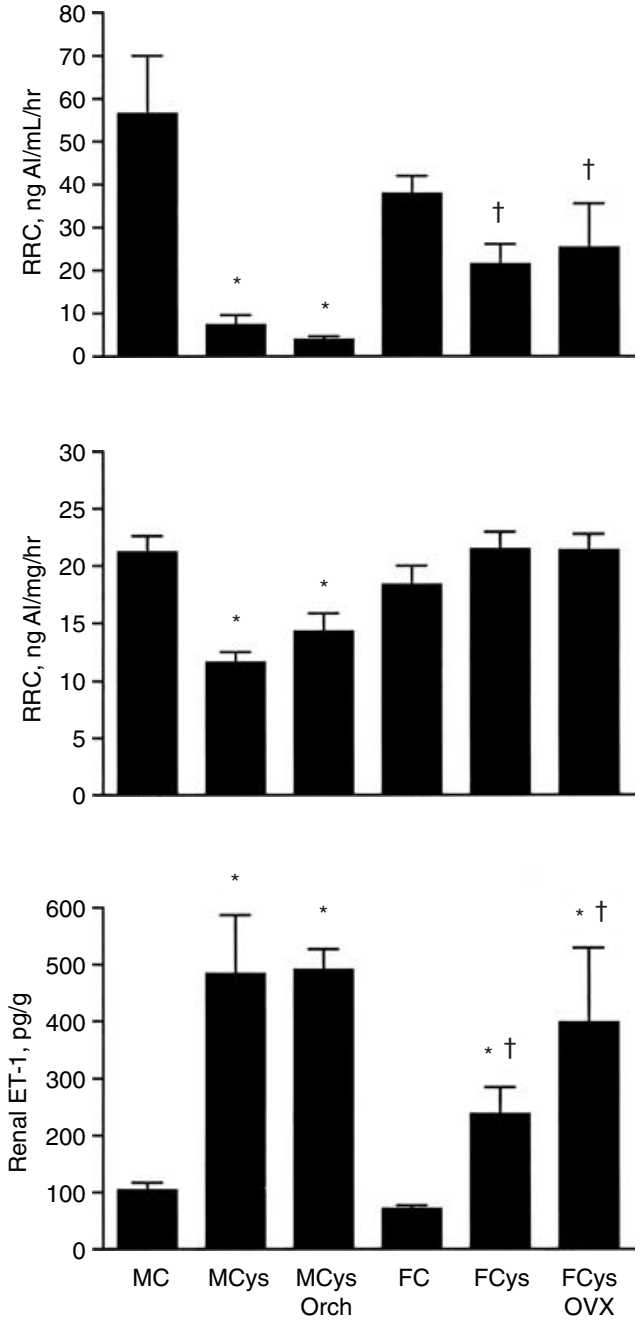

Fig. 5. Plasma renin concentration (PRC), renal renin concentration (RRC), and renal endothelin-1 (ET-1). PRC and RRC were suppressed in the cystic male groups, but not in the cystic female groups. Renal ET-1 expression was up-regulated in cystic males, and not affected by orchiectomy. In females, the degree of up-regulation associated with the cystic state was lesser than in males, and was attenuated by ovariectomy. $^{*}P < 0.01$ vs. respective control; $\dagger P < 0.05$ vs. respective male group.

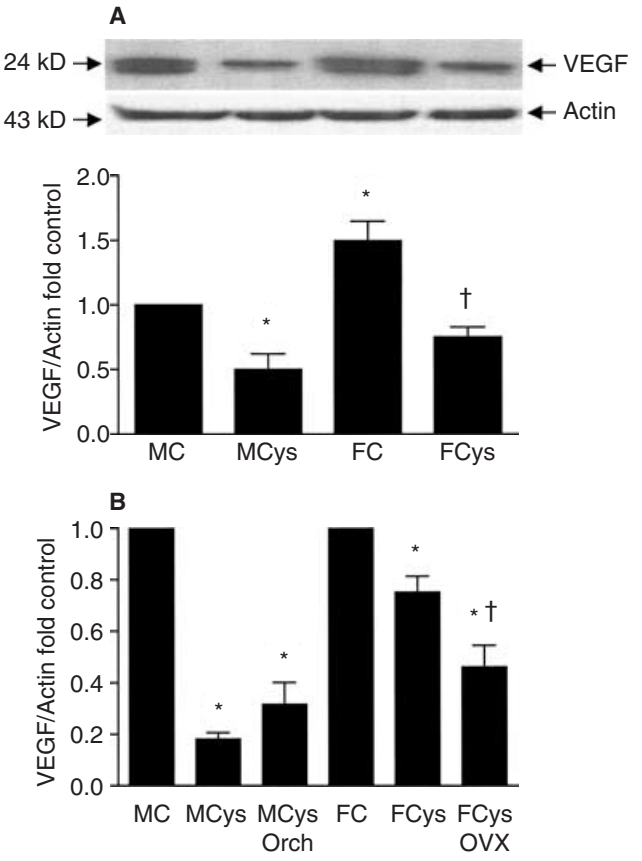

Fig. 6. Representative Western blots of vascular endothelial growth factor (VEGF) and actin in male and female, cystic and noncystic rats. (*A*) The VEGF/actin ratio was reduced in male cystic (MCys) rats, as compared with noncystic controls (MC). Noncystic females (FC) demonstrated higher expression than noncystic males, but the cystic phenotype (FCys) was also associated with reduction in VEGF expression in female rats. $^{*}P < 0.05$ vs. control males; $\dagger P < 0.01$ vs. control females. (*B*) Orchiectomy (Orch) led to a slight but not significant increase in VEGF; ovariectomy (OVX) significantly reduced renal VEGF expression. $^{*}P < 0.05$ vs. respective controls; $\dagger P < 0.01$ vs. cystic females.

onset of ESRD than do affected women [1–7, 19–21]. Similar gender dimorphism has been noted in the Han:SPRD [8–12] and pck [22] rat PKD models; data in mouse models are less clear. The present studies confirm prior observations of gender dimorphism in progression of experimental PKD. As others have shown [8–12], female rats are relatively protected against expression of the disease phenotype, with smaller kidneys, a lower cyst burden, and relative preservation of renal function. Though not yet studied in a PKD model, administration of exogenous estradiol has been reported to have renoprotective effects in experimental models of diabetes [23], aging [24], renal ablation [25], and chronic allograft nephropathy [26].

Renal function and cyst burden: Males

Prior gender studies assessed renal function with relatively insensitive methodologies (e.g., serum creatinine or creatinine clearance). As previously reported [16–18], more precise clearance measurements indicate intense renal vasoconstriction in the male rats, with decreased values for ERPF, and elevation of FF and RVR. These results correspond with clinical reports of low ERPF, high RVR, and elevation of FF [27–32], as well as severe

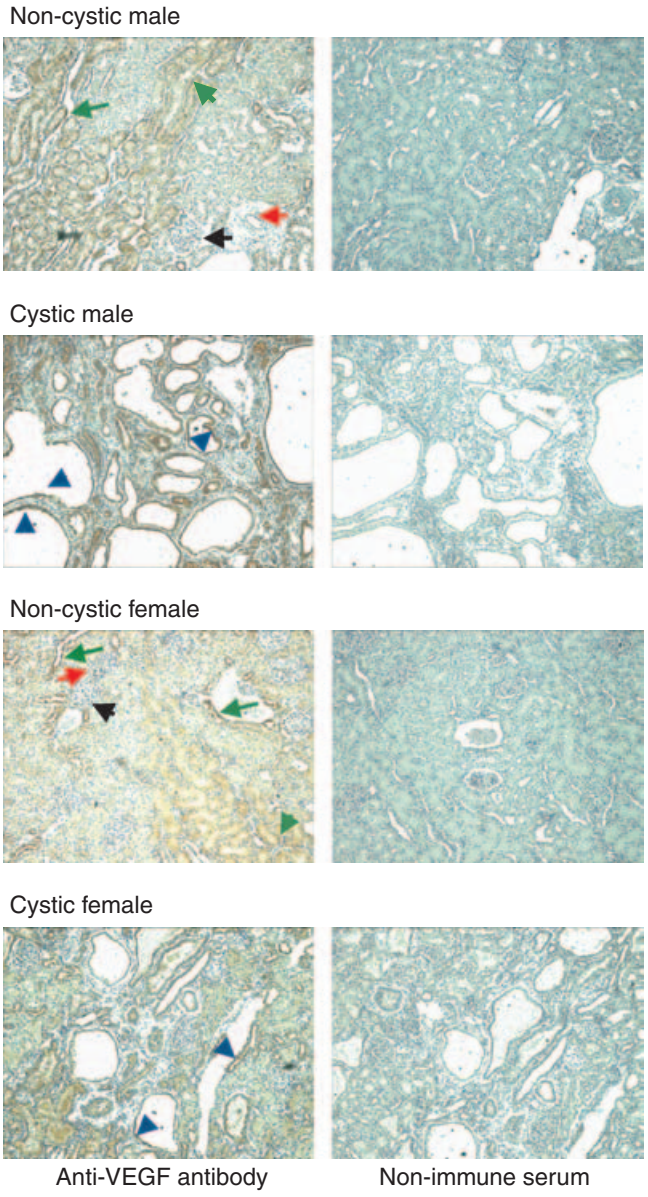

Fig. 7. Representative immunohistochemical expression of vascular endothelial growth factor (VEGF) in male and female, cystic and noncystic rats. Left panels show sections stained with anti-VEGF antibody. Right panels show parallel control sections incubated with nonimmune serum. In noncystic male and female kidneys, the VEGF was found predominantly in distal tubules (green arrows), glomerular epithelial cells (black arrows), and in some arterial or arteriolar endothelia (red arrows). In both male and female cystic rats, renal VEGF immunoreactivity was found mainly in epithelial cells lining the cyst walls as well in atrophic tubuli (blue arrows). There were no apparent differences between male and female rats.

sclerosis of the preglomerular vessels [33]. More recently, magnetic resonance measurements have confirmed strong concordance between reductions in renal blood flow and disease severity in patients with ADPKD [34]. Furthermore, renal blood flow declines earlier than GFR, and predicts structural and functional progression in clinical ADPKD [abstract; Torres V, et al, *J Am Soc Nephrol* 15:13A, 2004].

The gonadal ablation studies provided further evidence for a role of gender and/or related hormones in the modulation of renal hemodynamics. The renal vasoconstriction in male cystic rats was attenuated by orchiectomy, suggesting that intact androgen status contributes to renal insufficiency. Our inability to detect a favorable effect of orchiectomy on cyst burden was likely related to the relatively short period of observation, as well as to the relatively late age at which the gonadal ablation was performed. Cystic changes in this model are expressed very early in life, with significant increases in renal size seen in the first few weeks of life. At the time of orchiectomy, cystic changes were already in progress, and therefore effects of short-term intervention on kidney weight and cyst burden were likely more difficult to detect.

The equivalent values for GFR and ERPF (normalized for body weight) in the intact, noncystic male and female groups suggest a minimal influence of sex hormones on baseline renal function. Gender hormones have not been extensively studied in this model. Serum testosterone levels are low in cystic male rats [35], while there is increased renal expression of the androgen receptor, as compared with noncystic males [36]. However, the higher androgen receptor levels were located the cystic epithelium, where a role in modulation of renal blood flow would likely be indirect at best, and serum testosterone levels have not been well correlated with intrarenal events. Another abstract noted that administration of the androgen receptor antagonist flutamide was associated with slowing of cyst development, associated with suppression of elevated serum creatinine activity [36]. In addition, male cystic kidneys exhibit higher levels of macrophage chemoattractant protein-1 (MCP-1) than do female cystic kidneys [37]. It has also been suggested that androgens may contribute to apoptosis and thereby influence progression [38], but this pathway has not been well explored in vivo. Though not well studied in renal disease, androgens have the potential for interaction with important endogenous vasoactive mediators which may have contributed to the apparent negative effects (see below).

Renal function and cyst burden: Females

With the clearance methods in the present study, we are able to demonstrate preservation of normal renal function in the female cystic rats. In concert with the lower cyst burden, cystic females exhibited essentially normal values for GFR and ERPF. However, when factored for body weight, both GFR and ERPF fell after ovariectomy, suggesting that endogenous levels of estrogen and/or related female hormones contributed to protection against the development of renal vasoconstriction in this model.

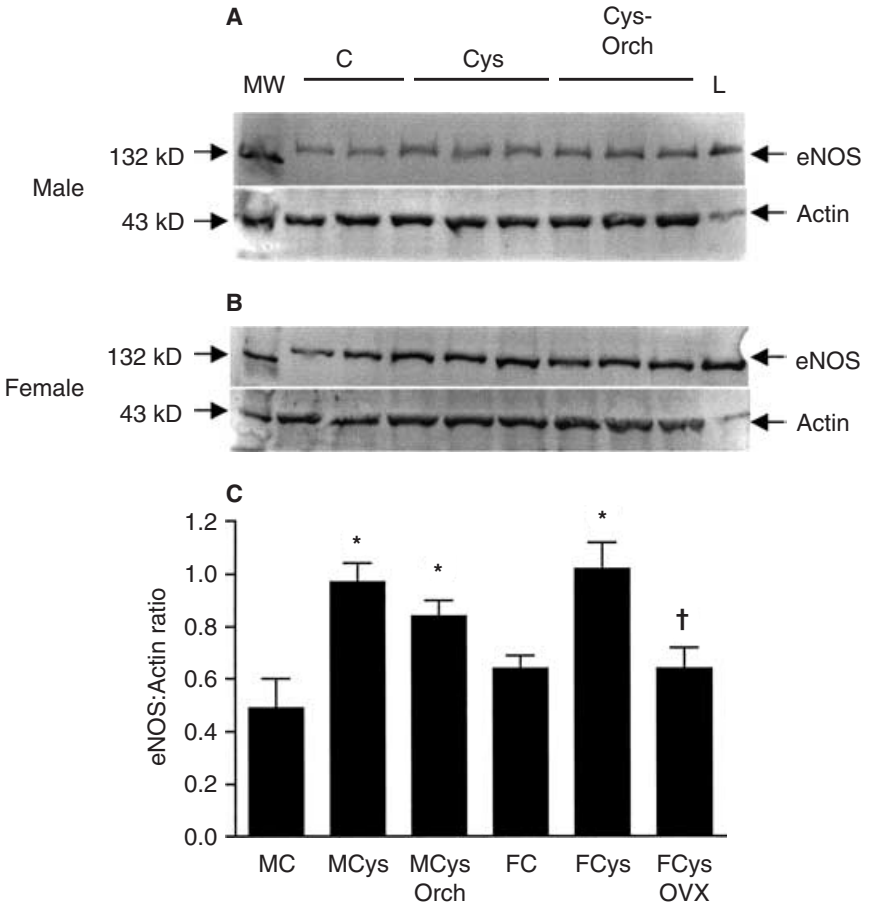

Fig. 8. Representative Western blots of endothelial nitric oxide synthase (eNOS) and actin in male (*A*) and female (*B*), cystic and noncystic rats. (C) The eNOS/actin ratio was increased in male cystic rats, as compared with noncystic controls, and not affected by orchiectomy (Orch). In females, the cystic state was associated with further up-regulation of eNOS, but this up-regulation was limited by ovariectomy (OVX). MW is molecular weight standard; L is human endothelial cell lysate positive control. $^{*}P < 0.001$ vs. respective noncystic controls; $\dagger P < 0.01$ vs. cystic females.

There is substantial evidence that estrogen plays a role in modulation of vascular function, in the kidney as well as in the systemic circulation. As is further discussed below, estrogens interact with a number of endogenous vasoactive mediators which may contribute to its apparent beneficial effects. In the Han:SPRD model, we found that intact, noncystic females had GFR and ERPF values (relative to body weight) that did not differ from those in males, so baseline (strain) differences did not play a role. Only when the cystic state was superimposed did the gender difference become expressed. On the other hand, ovariectomy produced a detrimental effect in cystic females, suggesting a protective role for estrogen and/or related hormones.

Role of the renin-angiotensin system (RAS)

These studies further examined activity of several vasoactive disease mediators, which may explain some of the sex hormone–associated differences. Prior studies have suggested that inappropriate or elevated activity of the RAS may be involved in regulation of renal function and cyst progression in ADPKD. Renal parenchymal ischemia, resulting from compression induced by cystic enlargement, has been postulated to stimulate the RAS. Studies in ADPKD patients have noted hyperplasia of the juxtaglomerular apparatus, and redistribution and expansion of renin immunoreactivity into blood vessels and the tubulointerstitium [39, 40], as well as in the cyst fluid [39]. Furthermore, angiotensin II (Ang II) formation independent from angiotensin converting enzyme (ACE) is suggested by the finding of increased chymase-like Ang II–generating capacity in human ADPKD kidneys [41]. Ectopic renin expression is postulated to contribute to increased renal Ang II production. Indeed, all RAS components are found in the cyst apparatus, suggesting the possibility of an autocrine/paracrine RAS in the cystic kidney [42]. In further support of the hypothesis of intrarenal RAS activation are observations of low PRC (presumably suppressed by intrarenal RAS activation), and exaggerated renal vascular responsiveness to RAS blockade [16].

In the present studies, we again found suppression of PRC in the cystic male rats; orchiectomy had no apparent effect. RRC was also suppressed in cystic males. Though not well studied, androgens if anything serve to stimulate the RAS [43], but we could not confirm that postulate with the present study design. The low RRC values in cystic males may be a consequence of progressive loss or destruction of the juxtaglomerular apparatus due to

cyst formation. Furthermore, suppression of RRC could be a consequence of higher intrarenal Ang II levels, but without localization data, no further conclusions can be drawn. In the female rats, PRC and RRC in intact noncystic females did not differ from values in noncystic males. In contrast to the males, there was no apparent effect of the cystic state on either parameter, nor any effect of ovariectomy. The relationship between estrogen and the RAS is complex. Estradiol increases angiotensinogen, while suppressing renin, ACE, aldosterone, and the angiotensin type 1 (AT_1) receptor [44–48], and up-regulates the AT_2 receptor [48]. On the other hand, some estrogen preparations such as those in oral contraceptives have been reported to stimulate the RAS [49]. Future studies will be required to dissect the interaction between estrogen and the RAS in this model.

Role of ET-1

The RAS in turn interacts with many other mediators, which are also influenced by sex hormones. ET-1 is a potent vasoconstrictor which is implicated in the pathophysiology of cyst formation and renal dysfunction in this model [18, 50]. Plasma ET-1 levels are high in ADPKD patients [51, 52], and ET-1 is present in human cyst fluid [51]. Serum ET-1 levels are higher in men than in women [53], and estrogen therapy [such as hormone replacement therapy (HRT)] reduces ET-1 levels [54]. Furthermore, estradiol inhibits basal and Ang II–induced ET-1 synthesis in vascular endothelial cells [55]. As in prior studies [18], we again found marked elevation of renal ET-1 levels in kidneys from cystic males; orchiectomy was without effect. There were no significant differences between male and female noncystic kidneys. Cystic female kidneys exhibited increased expression of ET-1, though not to the degree seen in males, suggesting a modulatory effect of gender. When this modulatory effect of gender was abrogated by ovariectomy, renal ET-1 levels rose toward levels near those seen in cystic males. Thus, ET-1 appears to be a significant disease mediator; recent evidence suggests that ET-1 is detrimental via the ET_B receptor [56]. Limitation of ET-1 in female rats (presumably at least in part by estrogen) may contribute to the amelioration of renal vasoconstriction, as well as to the lesser development of cystic injury.

Role of nitric oxide

The RAS, ET-1, and sex hormones also interact with nitric oxide. Estradiol is a potent stimulus for formation of eNOS and subsequent generation of nitric oxide [57]. Clinically, HRT increases nitric oxide, suppresses ET-1, and thus increases the nitric oxide/ET ratio [54]. Female rats exhibit higher endogenous renal eNOS mRNA and protein, and lesser vasoconstriction in response to NOS inhibition [58], while male rats are more prone to proteinuria after NOS blockade [59]. Effects of androgens on nitric oxide in the kidney are less well studied, though testosterone has variable effects on vascular reactivity in different experimental systems.

We reported earlier that in renal eNOS was up-regulated, in male cystic rats, but primarily in extravascular structures [18]. Our data were consistent with the scenario of exaggerated hemodynamic actions of Ang II due to both loss of buffering by a dysfunctional nitric oxide system, and by concomitant activation of the ET system. With regard to eNOS expression, we again found evidence of up-regulation in cystic male kidneys, as compared with noncystic males. Orchiectomy had only a slight effect on renal eNOS expression. As compared with males, however, eNOS levels were comparably up-regulated in cystic females, but then reduced by ovariectomy, suggesting that eNOS is at least in part regulated by estrogen in the cystic kidney. It should be also noted that determination of sole eNOS protein expression does not fully reflect its enzymatic activity and resulting protective potential of eNOS-derived nitric oxide. The present findings should prompt future studies in PKD focusing on functional characteristics of eNOS, such as phosphorylation and coupling-uncoupling status.

Role of VEGF

All of the above hormones and mediators also interact with VEGF, a mediator with a complex role in the kidney [60]. Estrogen is a potent stimulator of VEGF and angiogenesis [61]. Little is known of VEGF in PKD, though it has been postulated that ischemia from cyst compression, and resultant hypoxia, might represent local stimuli for VEGF expression. Bello-Reuss et al [62] found up-regulation of VEGF in the vicinity of the cysts in human ADPKD kidneys, and postulated that angiogenesis and enhanced capillary formation support the perpetuation of cyst growth. Recent abstracts have noted increased renal expression of the angiogenic isoforms of VEGF [abstract; Tao Y et al, *J Am Soc Nephrol* 14:582A, 2004], and high levels of VEGF (by ELISA) in kidneys and in cyst fluid of Han:SPRD rats, with reduction of cyst volume density after combined VEGFR1 and 2 blockade [abstract; Tao Y et al, *J Am Soc Nephrol* 15:656A, 2004]. While there is evidence of a detrimental effect of VEGF in glomerular pathophysiology and possibly cyst growth, its role in preservation of the tubulointerstitium appears to be more positive. Kang et al [63, 64] have demonstrated an association between VEGF deficiency and peritubular capillary loss and development of fibrosis in renal disease models. In studies of the remnant kidney, these investigators demonstrated relative VEGF deficiency in male kidneys, exacerbated by the superimposition of reduced

renal mass [64]. Those findings are in complete accord with our observations of down-regulation of VEGF in kidneys of cystic males, as compared with cystic females. A role for androgens is suggested by downregulation of VEGF in kidneys of noncystic males, as compared with females, and with exaggerated suppression of VEGF in cystic males, as compared with cystic females. On the other hand, ovariectomy was associated with reduction in VEGF in cystic female kidneys. Taken together, these data are suggestive of a protective role of VEGF (and of estrogen-associated VEGF expression) in the cystic kidney. Further studies, designed to examine the peritubular capillaries, would be of interest in this regard.

The present findings expand upon prior observations of gender dimorphism in this model, and suggest a prominent role for gender hormones. We speculate that in the males, the presence of intact androgen status is associated with stimulation of the RAS and ET-1 systems. Down-regulation of VEGF and possibly bioavailable nitric oxide further contribute to vasoconstriction and progression of cystic and fibrotic disease components. In the females, estrogen has a protective effect, inducing suppression of the intrarenal RAS and ET-1 systems, and up-regulation of VEGF, thereby promoting preservation of renal function and attenuation of the loss of structure. The estrogen effect may be more dominant than the androgen effect. Further studies of specific gender hormone modulation will be of interest, in search of clinically applicable approaches to slowing the progression of ADPKD.

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Reprint requests to Sharon Anderson, M.D., Division of Nephrology and Hypertension, PP262 Oregon Health and Science University, 3314 SW US Veterans Hospital Rd., Portland, OR 97239-2940.
E-mail: anderssh@ohsu.edu

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